# Inducible Nitric Oxide Synthase in Circulating Microvesicles: Discovery, Evolution, and Evidence as a Novel Biomarker and the Probable Causative Agent for Sepsis

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**Background:** The sepsis pathology remains an enormous medical problem globally because morbidity and mortality remain unacceptably high in septic patients despite intense research efforts. The economic and societal burden of sepsis makes it the most pressing patient care issue in the US and worldwide. Sepsis is a dysregulated immune response normally initiated by an infection. The need for an early, accurate, and reliable biomarker test to detect the onset of sepsis and for a targeted sepsis therapy is widely recognized in the biomedical community.

**Content:** This report reviews the published findings relevant to microvesicle-associated inducible nitric oxide synthase (MV-A iNOS) as a novel plasma biomarker for the onset of sepsis, including human clinical studies and animal studies. Plasma iNOS as both a standalone test and 1 of the components of a novel panel of biomarkers to stage the progression of sepsis is presented and discussed in comparison with other biomarkers and other proposed panels of biomarkers for sepsis.

**Summary:** The data strongly support the concept that extracellular plasma MV-A iNOS in circulating microvesicles is centrally involved in the initiation of sepsis, and a diagnostic test based on plasma iNOS can serve as an early presymptomatic warning signal for the onset of sepsis. A novel panel of plasma biomarkers comprising iNOS, pro-interleukin-18, pro-interleukin-33, and regenerating protein 1-α is proposed as a multianalyte presymptomatic method to stage the onset of sepsis for improved, prompt, data-driven patient care.

## IMPACT STATEMENT

The patient populations that will benefit most from an early, accurate, and reliable test for the onset of sepsis include trauma, surgical, and burn patients, as well as those in intensive care units; neutropenic, febrile cancer patients who are undergoing chemotherapy and/or radiation therapy; and individuals who visit the emergency room and are at risk for developing community-acquired sepsis. Plasma inducible nitric oxide synthase (iNOS) is an underappreciated and understudied biomarker for sepsis, and circulating extracellular microvesicle-associated iNOS is a novel candidate therapeutic target to treat sepsis. The goal is to review the research studies regarding the role iNOS plays in the initiation of sepsis.

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The sepsis pathology remains an enormous medical problem globally despite intense research efforts for >3 decades, during which legacy pharmaceutical and biopharmaceutical companies and government-supported research facilities and institutes worldwide have invested billions of dollars trying to solve the enigma of sepsis, with little success (*1*). The economic and societal burden of sepsis makes it the most pressing patient care issue in the US and worldwide. Nonhospital secondary costs for sepsis exceeded $20 billion in 2013 in the US (*2*). Sepsis is a clinical condition caused by a dysregulated immune response to infection, and as sepsis progresses, multiorgan dysfunction often occurs. Morbidity and mortality remain unacceptably high in septic patients. Mortality has been reported as high as 40% in hospital settings and increases after discharge because of the long-term health complications of sepsis (*3*).

Even defining what constitutes sepsis has proven to be difficult as is illustrated by the 3 definitions of sepsis. After international conferences in 1992 (*4*), 2001 (*5*), and 2016 (*6*), a consensus definition has been established. The current consensus definition, Sepsis-3, was published in 2016. One of the major problems is different patients who clearly have sepsis do not display the same symptoms as other septic patients. Because a spectrum of symptoms exists, an all-encompassing definition based on symptomology including the presence of an infection has been difficult to achieve.

The clinical need for a specific and reliable in vitro diagnostic (IVD)[4] test based on a biomarker or panel of biomarkers for detecting the onset of sepsis early is widely recognized and well documented (*7*–*12*). Most clinicians in this field agree an IVD test for the sepsis pathology would be a major breakthrough and provide important information about a patient's physical status for improved, prompt, data-driven best practices treatment and care.

In 2012, this need was highlighted in a headline news story published by *The New York Times* (*13*). Rory Staunton was a 12-year-old boy who 4 days after scraping his arm, after visits to his doctor and hospitals, and after multiple misdiagnoses, died of cardiac arrest as the result of severe sepsis. After Rory's death, new regulations were enacted in the state of New York mandating sepsis screening for all individuals who visit a hospital emergency room (*14*, *15*). Based on increased awareness of sepsis and mandatory sepsis screening, the mortality rate for sepsis in the state of New York has decreased but remains unacceptably high (*16*). Numerous potential biomarkers for sepsis have been investigated (*9*–*12*). At present, the 3 most ordered IVD tests for sepsis are L-lactate, C-reactive protein, and procalcitonin (PCT). Other candidate biomarkers for sepsis include heparin binding protein (*17*, *18*), presepsin, which is also known as soluble CD14 subtype (*19*–*22*), and CD64 (*23*–*26*). A novel form of the intracellular enzyme inducible nitric oxide synthase (iNOS) that is not normally found in blood has been investigated

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[4] **Nonstandard abbreviations:** IVD, in vitro diagnostic; PCT, procalcitonin test; iNOS, inducible nitric oxide synthase; MV-A, microvesicle-associated; NO, nitric oxide; NOS, nitric oxide synthase; mAb, monoclonal antibody; EIA, enzyme immunoassay; ICU, intensive care unit; P-7/8, patient 7/8; SIRS, systemic inflammatory response syndrome; PBMC, peripheral blood mononuclear cell; ER, emergency room; pro-IL-18, pro-interleukin-18; pro-IL-33, pro-interleukin-33; Reg1α, regenerating protein-1α.

the past few years. During the onset of sepsis, iNOS is found in circulating microvesicles as microvesicle-associated (MV-A) iNOS in whole blood and plasma samples (*27*, *28*). Based on current research, circulating MV-A iNOS appears to play a major role in the onset of the sepsis cascade by causing cell damage and death that leads to organ dysfunction, one of the hallmarks of sepsis. This report will review plasma iNOS as an early and specific biomarker for the onset of sepsis.

## BACKGROUND ON NITRIC OXIDE AND THE 3 NITRIC OXIDE SYNTHASE ENZYMES

Research investigating nitric oxide (NO) as an effector molecule and the nitric oxide synthase (NOS) isozymes (EC 1.14.13.39) that produce NO has been active for >30 years. In 1992, NO was "Molecule of the Year" by *Science* (*29*), and in 1998, the Noble Prize in Physiology or Medicine was awarded to the 3 researchers who discovered the biological action of NO and its production (*30*). Over the years, >100 000 research articles have been published regarding NO and the 3 NOS enzymes that produce NO.

Three mammalian NOS enzymes exist: neuronal NOS (or NOS-1), iNOS (or NOS-2), and endothelial NOS (or NOS-3). Most enzymologists estimate iNOS can produce 500 to 1000 times more NO per minute than either neuronal NOS or endothelial NOS. Thus, iNOS is characterized as a high-throughput enzyme to produce large (toxic) quantities of NO. Although all 3 NOS enzymes catalyze the same reaction, they have different physiologic functions: neuronal NOS is involved in neurotransmission; endothelial NOS is involved in blood pressure regulation, homeostasis, and smooth muscle relaxation; and iNOS is involved mainly in host defense. The areas of research focus for iNOS are primarily inflammation (especially as it relates to sepsis and inflammatory bowel diseases), host defense, rheumatoid arthritis, and transplant rejection.

### The discoveries of plasma iNOS and MV-A iNOS

Monoclonal antibodies (mAbs) and immunoassays specific for human iNOS have been developed and characterized, including a 2-site enzyme immunoassay (EIA) (*28*, *31*, *32*). The capture mAb binds to residues 25 to 54 in the N-terminal oxygenase domain of human iNOS, and the labeled detection mAb binds to residues 985 to 1002 in the C-terminal reductase domain (*31*, *32*).

*Clinical trial #1 for plasma iNOS on intensive care unit (ICU) patients.* Because iNOS had been speculated to be involved in sepsis even though this normally intracellular enzyme was not expected to be found outside of cells, a small exploratory clinical study was conducted in which 10 ICU patients were enrolled—6 with confirmed sepsis and 4 without sepsis at enrollment. All clinical studies were approved by the Institutional Review Board. This first clinical trial was designed to test the sandwich EIA's ability to detect iNOS in plasma samples. On the day of enrollment, iNOS was present in plasma samples obtained from all 6 septic ICU patients and was absent from 3 of the 4 samples from nonseptic ICU patients. Contrary to clinical expectations, patient #7 (P-7), who was enrolled as a nonseptic study participant because he was not displaying any symptoms of systemic inflammatory response syndrome (SIRS; presepsis) or sepsis, was found to have iNOS in his plasma sample by Western immunoblot analysis (Fig. 1A), and iNOS could be measured in his plasma with the iNOS sandwich EIA (Fig. 1B, light gray bars). The intensivists did not suspect P-7 was becoming septic until day 2, when a blood sample was drawn for lab culture and antibiotic therapy was started. Thus, 48 h before the intensivists suspected the onset of sepsis, iNOS could be detected and measured in P-7's plasma by Western blots and

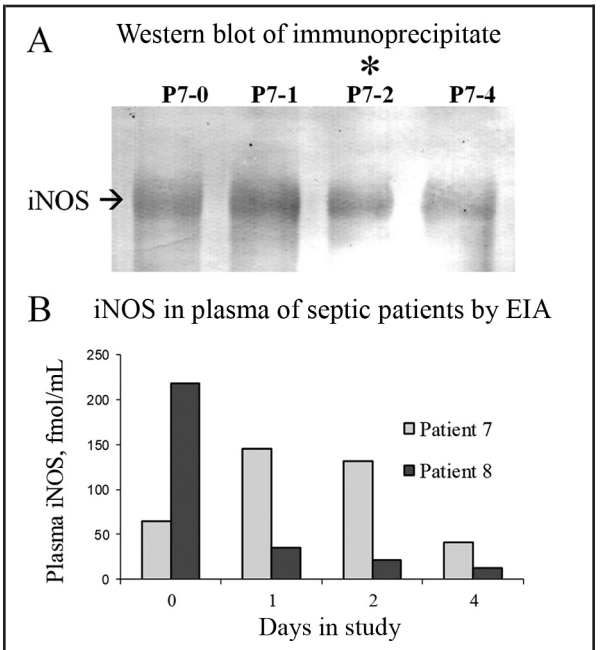


**Fig. 1. Discovery of iNOS in plasma.**
(A), Western immunoblots of immunoprecipitated iNOS from P-7 day 0, 1, 2, and 4 plasma in the first ICU study. (B), Concentration of iNOS measured by the EIA in plasma from P-7 (light bars) and P-8 (dark bars) on days 0, 1, 2, and 4 of the study (*27*, *28*). Asterisk denotes the day the intensivists first suspected sepsis.

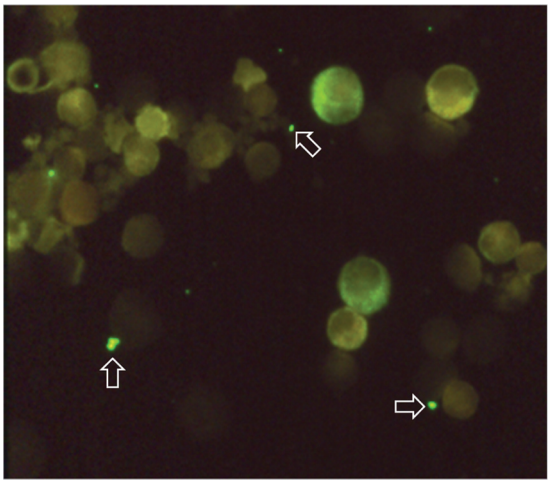

**Fig. 2. Discovery of MV-A iNOS.**
MV-A iNOS from a septic patient in a PBMC preparation immunostained with fluorescein isothiocyanate-labeled anti-iNOS mAb (*28*, *38*). The arrows are pointing to fluorescently immunostained, extracellular MV-A iNOS in a field containing both immunostained and unstained blood cells. These iNOS-containing microvesicles are found only in septic patients. The PBMC preparation was isolated by density gradient centrifugation.

the sandwich EIA. Patient #8 (P-8) was enrolled as a confirmed septic patient, and iNOS could be measured in her plasma with the EIA on the day of enrollment (Fig. 1B, black bars), and as effective therapy was applied, the plasma level of iNOS decreased. In the 4 completed clinical studies on ICU patients combined, the onset of sepsis was detected 24 to 48 h before the appearance of the physiological symptoms of sepsis in >150 study participants.

*Clinical trial #2 on ICU patients.* Because the sandwich EIA could detect and measure iNOS in plasma samples and because plasma iNOS appeared potentially to be a specific biomarker for the onset of sepsis based on this small sample size trial, a larger second clinical study was conducted in which 47 ICU patients and 11 healthy individuals were enrolled (see Table 1 in the Data Supplement that accompanies this article at http://www.jalm.org/content/vol3/issue4). As in the first clinical study, plasma iNOS was again found in blood samples 24 to 48 h before the appearance of the physiologic symptoms of sepsis (see Table 2 in the Data Supplement). During this study, whole blood samples were also collected and processed to prepare peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation to investigate the induction of iNOS in PBMCs. However, when the floating band of PBMCs was isolated and fluorescently immunostained with fluorescein isothiocyanate-labeled anti-iNOS mAbs, small fluorescent dots were observed, in addition to fluorescently stained and unstained PBMCs (Fig 2). At first, these were thought to be artifacts of the preparation. However, after studying the literature, these were eventually determined to be iNOS-containing microvesicles. To determine where and

how they were formed, more experiments and clinical studies were conducted.

## PLASMA iNOS AND THE ROLE MV-A iNOS PLAYS IN SEPSIS

More than a year after the first public presentation describing the discoveries of plasma iNOS and MV-A iNOS (*28*), published reports from other groups of investigators started to appear confirming and extending these initial observations. In 3 publications, Han and associates demonstrated active iNOS was required for hepatic (*33*), pulmonary (*34*), and intestinal epithelial tight junction (*35*) dysfunction in endotoxemic mice used as an animal model of sepsis. In this series of reports, these investigators recognized that active iNOS was required to produce the leakage they observed through the barrier structures, but they were not able to determine how active iNOS was exerting its effect(s). More recent data strongly support the concept that active iNOS was transported to these cells in microvesicles, and the iNOS enzyme was intercalated into receiver cells in these barrier structures. After entering susceptible receiver cells, the iNOS enzyme produced toxic quantities of NO and peroxynitrite inside the cells, which killed the cells and resulted in microperforations in, as well as leakage through, the cellular barriers observed by Han and associates.

In publications from Dr. Peter Brouckaert's research team at Ghent University, these investigators, through the use of 6 different animal models of sepsis, convincingly demonstrated (*a*) iNOS is centrally involved in the sepsis pathology, (*b*) there is "good" iNOS that is intracellular and protects cells from oxidative stress and "bad" iNOS that is extracellular and causes cellular damage, and (*c*) the "bad" iNOS originates from parenchymal cells, not from cells of hematopoietic lineage (*36*, *37*). They also effectively disproved the concept that white blood cells produce excess NO during septic shock (*37*).

At other conferences, presentations were made during which a novel hypothesis and early data were presented that strongly suggested circulating MV-A iNOS could be a new therapeutic target to treat sepsis before organ dysfunction (*38–40*). These presentations included data showing MV-A iNOS is lethal when administered to healthy animals and demonstrated the ability to intervene in 2 different animal models of sepsis with an in vivo neutralizing anti-MV-A iNOS mAb, and thereby rescue up to 80% of the challenged animals from death by sepsis (*41*). Subsequently, Gambin et al. (*42*) demonstrated iNOS-containing microvesicles (exosomes) were found in, and could be isolated from, the plasma of all septic patients enrolled in their clinical study, but they were not present in plasma samples from nonseptic patients or normal controls. These investigators showed iNOS-containing microvesicles fused with cultured endothelial cells; the iNOS was internalized into the cells; and the internalized iNOS enzyme produced toxic quantities of NO and peroxynitrite. These processes resulted in the death of the cultured endothelial cells (*42*). The microvesicles isolated from healthy study participants and nonseptic ICU patients had no effect on the cultured endothelial cells because they did not contain MV-A iNOS. Also, Azevedo et al. (*43*) found circulating iNOS-containing microvesicles, as exosomes, in all the septic patients studied, and the isolated iNOS-containing microvesicles caused hemodynamic collapse of perfused naïve rabbit hearts. Microvesicles isolated from every septic ICU study participant contained MV-A iNOS, whereas the microvesicles isolated from healthy humans and nonseptic ICU patients did not contain iNOS. The iNOS contained in the circulating microvesicles isolated from septic patients was transferred into the cardiomyocytes of perfused naïve rabbit hearts, was enzymatically active, and produced toxic quantities of NO and peroxynitrite.

This led to cardiac dysfunction and the hemodynamic collapse of the perfused hearts by a process almost identical to that which occurs when humans die of septic shock (*43*). Later, Mortaza et al. (*44*) induced sepsis in rats by cecal ligation and puncture. They isolated microvesicles from normal, sham-operated, and septic rats, and dosed healthy rats with the 3 different purified microvesicle preparations. The microvesicles isolated from normal and sham-operated rats had no effect when administered to the healthy recipient rats. However, the iNOS-containing microvesicles isolated from the septic rats caused hypotension in the healthy recipient rats because of the overproduction of NO by the MV-A iNOS, which was transferred to the aorta and heart in the recipient rats. This ultimately led to hemodynamic collapse in the recipient rats and to their deaths (*44*). Thus, these groups of investigators confirmed the original findings (*38–41*) that MV-A iNOS is lethal, can enter susceptible receiver cells, and, once inside the receiver cell, produces toxic quantities of NO that causes cell damage and death and results in organ dysfunction (*42–44*).

Collectively, these data strongly support the concept that circulating extracellular MV-A iNOS leads to the onset of the sepsis cascade. During the investigation of plasma iNOS as a potential biomarker for sepsis (*27*, *28*, *31*), circulating extracellular microvesicles have gone from platelet dust and remnant cell debris (*45*) to being recognized as integral parts of normal cell-to-cell communication by transporting cargo from one cell to another cell (*46*). However, microvesicles are also thought to play important roles in numerous pathophysiological processes, including cardiovascular disease (*47*, *48*), cancer metastases (*49*, *50*), and sepsis (*28*, *38–44*). Traditionally, microvesicles were isolated by ultracentrifugation for hours to pellet them before analysis, but more recently, they have been isolated by ultrafiltration, membrane absorption, and immunocapture procedures.

## PROPOSED MECHANISM OF ACTION OF MV-A iNOS

As the understanding of the transport of macromolecules, such as MV-A iNOS, as cargo by circulating microvesicles grew and was integrated into the current understanding of the sepsis pathology and the published data, a new model for the onset of sepsis and the role MV-A iNOS might play in the pathophysiology started to emerge (Fig. 3) (*38–41*). While the iNOS-containing microvesicles are in the circulatory system, iNOS is an inactive enzyme (see Table 3 in the Data Supplement) because of the lack of 2 required cofactors. However, once the circulating extracellular iNOS-containing microvesicles are internalized into susceptible receiver cells, the MV-A iNOS becomes an active enzyme because all its required substrates and cofactors are present inside the receiver cell. However, the iNOS enzyme is now a component of a cell that has never been induced; it is in an inappropriate location; and the vulnerable receiver cell is out of normal cellular regulation. Once inside receiver cells, the iNOS enzyme produces toxic quantities of NO and peroxynitrite that results in the death of the receiver cell and, depending on where this damage occurs, leads to the formation of microperforations in the blood–brain barrier, the tight junctions of the intestine, the glomerular filtration units of the kidney, and the capillary beds of the circulatory system, as well as ultimately to leakage through one or more of these barrier structures. It can also lead to damage of cardiomyocytes in the heart and result in hemodynamic collapse. These are the classic symptoms of sepsis, severe sepsis, and septic shock.

### Plasma iNOS as a biomarker for the onset of sepsis in the ICU

*Clinical trial #3 on ICU patients.* Originally, it was hypothesized that the detection of iNOS in plasma could be used as an early warning signal for the

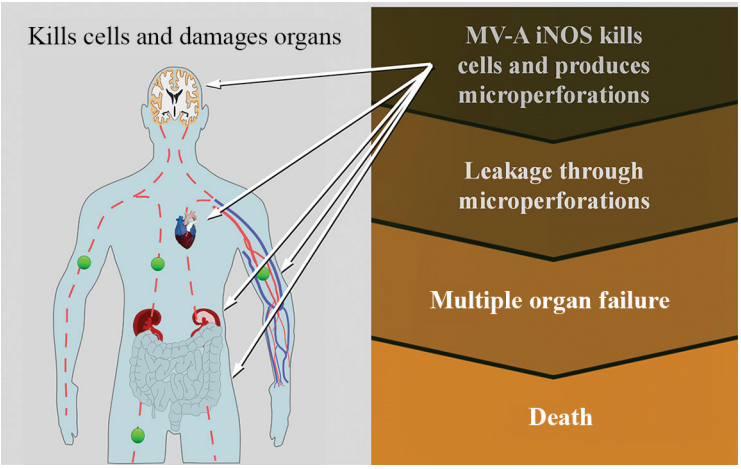


**Fig. 3. MV-A iNOS role in the sepsis cascade.**
This diagram illustrates the role MV-A iNOS, shown as green balls, plays in the sepsis cascade (*38*, *40*). Once the MV-A iNOS is delivered to susceptible cells, it is internalized, produces toxic quantities of NO and peroxynitrite inside the vulnerable receiver cell. This can kill the cell, produce microperforations in barrier structures, and lead to hemodynamic collapse.

onset of sepsis that would lead to aggressive therapy and improved patient outcomes. Previously, in clinical studies #1 and 2 of this project, it was demonstrated in a limited number of ICU patients and healthy individuals that the normally intracellular enzyme iNOS could be detected and measured in plasma obtained from ICU patients who were at risk of becoming septic or who were already septic. The results obtained from both earlier clinical studies were encouraging. Thus, the next step in this project was to determine in a statistically significant number of patients and healthy controls whether the level of iNOS in plasma can be used as a reliable early biochemical marker for the onset of sepsis (see Tables 4 and 5 in the Data Supplement). A total of 274 individuals were enrolled in clinical study #3—238 ICU study participants and 36 healthy volunteers—and they were stratified into 4 groups of study participants. The ICU patients were classified into 1 of 3 groups based on their physical status on the day of enrollment. Group #1 included SIRS-positive study participants; group #2 comprised confirmed septic study participants; group #3 included study participants who were neither SIRS positive nor septic but who were at risk for developing sepsis; and group #4 study participants were healthy volunteers who had no apparent ongoing illness. Of the 95 SIRS-positive study participants in group #1, 28 developed sepsis during the study period, and iNOS was found in the plasma of all these study participants 1.97 ± 1.13 days in advance of a blood sample being drawn for culture. Of the 51 septic study participants in group #2, iNOS was detected in the plasma of 43 study participants, and the 8 study participants in whom iNOS was not detected had all been treated with antibiotics before their enrollment in this clinical study. Of the 92 study participants at risk of becoming septic in group #3, 20 did develop sepsis during the study period, and iNOS was found in the plasma of all of these study participants 1.31 ± 1.13 days in advance of a blood sample being drawn for lab culture. None of the healthy volunteers gave a positive plasma iNOS result. Confounding the results of this study are 36 group #1 and 31 group #3 study participants who were found to be iNOS positive, but none of these participants had lab culture work performed as part of their hospital care. Many of them were on

**Table 1. Plasma iNOS as a predictor for the onset of sepsis-associated organ dysfunction in ICU patients.**

| | Heart, lung, or kidney dysfunction[b] | |
|---|---|---|
| n = 187[a] | Present | Absent |
| iNOS | | |
| Positive | 107 | 5 |
| Negative | 15 | 60 |
| Sensitivity = 88% | PPV[c] = 96% | |
| Specificity = 92% | NPV[d] = 80% | |

[a] 151 ICU trauma patients and 36 normal healthy individuals.
[b] The assumptions used: mean arterial pressure <70 mmHg or the administration of vasopressors constitutes hemodynamic dysfunction; blood urea nitrogen >20 mg/dL constitutes renal dysfunction; and diagnosis of respiratory failure, mechanical ventilation for >24 h or synchronized intermittent mechanical ventilation with changes in blood gasses and pH constitute pulmonary dysfunction.
[c] Positive predictive value.
[d] Negative predictive value.

anti-infectives, but it is unknown whether they were suspected of being septic or were being administered anti-infectives as a prophylactic treatment.

When a subgroup analysis of the 151 study participants who were ICU trauma patients enrolled in this study was performed, the results were significant (Table 1). The plasma iNOS test could forecast organ dysfunction in the heart, lung, or kidneys before the symptoms of sepsis appeared. These analyses focused on predicting organ dysfunction associated with the sepsis pathology using plasma iNOS as the biomarker. The results of these analyses showed that the plasma iNOS EIA has a sensitivity of 88%, a specificity of 92%, a positive predictive value of >96%, and a negative predictive value of 80% for organ dysfunction associated with sepsis in this ICU patient population. The sensitivity and specificity results are remarkable because it has been shown that in the absence of a gold standard test, the highest values achievable with a new IVD test are approximately 90% (*51*, *52*). Thus, these results are approaching the theoretical limits.

In advance of starting a pivotal clinical study to obtain regulatory agency marketing clearance for the plasma iNOS EIA as a biomarker for the onset of sepsis, 2 additional preliminary clinical studies were conducted. The first established the normal human reference range of plasma iNOS (*53*) by assaying 2 different sample volumes in triplicate of 100 normal human plasma samples. The results showed 99 of the samples to be below the level of detection of the chemiluminescent plasma iNOS EIA test, and 1 sample gave an extremely high readout. This sample was investigated using several assays for iNOS and for potential heterophilic interactions. It was shown not to contain iNOS, human antimouse antibodies, or 3 additional heterophilic materials. The cause of the spurious assay result was never determined, but it was not iNOS in the plasma sample. The second study set the cutoff concentration for plasma iNOS in ICU patients. This was achieved with 40 plasma samples obtained from confirmed early septic ICU patients and 40 plasma samples obtained from non-SIRS/nonseptic ICU patients as study participants. The 80 samples were assayed in triplicate at 2 different sample volumes. When using a cutoff value of iNOS positive at ≥1.5 ng/mL (≥5 fmol/mL) for ICU patients, all 40 of the early septic ICU study participants were iNOS positive, and 37 of the 40 non-SIRS/nonseptic ICU study participants were iNOS negative and 3 were iNOS positive. Although no follow-up clinical data are available, these 3 individuals may have been presymptomatic but already in the early stages of becoming septic because this test has been shown to yield positive results 24 to 48 h before the appearance of symptoms during previous clinical studies.

## Plasma iNOS as a biomarker for the onset of sepsis in the emergency room

*Emergency room (ER) clinical trial cohort #1.* The story of Rory Staunton and his misdiagnosis mentioned earlier is not an isolated case as is illustrated by the data collected during 2 clinical studies of plasma iNOS as a biomarker for the onset of sepsis in ER patients as study participants. In

**Table 2. Comparison of plasma iNOS to procalcitonin as a biomarker to detect the onset of sepsis in ER cohort 1.**

| n = 146 | Plasma iNOS results[a] | | n = 141 | PCT results[b] | |
|---|---|---|---|---|---|
| iNOS | Septic | Nonseptic | PCT | Septic | Nonseptic |
| Positive | 76 | 12 | Positive | 73 | 32 |
| Negative | 14 | 44 | Negative | 28 | 8 |

[a] Sensitivity, 84.4; specificity, 78.5; positive predictive value, 86.3; negative predictive value, 75.8.
[b] Sensitivity, 72.3; specificity, 20.0; positive predictive value, 69.5; negative predictive value, 22.2.

collaboration with Dr. Alan Wu and his research team at the University of California, San Francisco and the Zuckerberg San Francisco General Hospital, the utility of plasma iNOS as a biomarker for the onset of sepsis in an ER population was investigated (*54*). In the initial discovery cohort of 146 ER study participants, the plasma iNOS EIA test was assessed for its ability to detect extracellular plasma MV-A iNOS as a biomarker for the onset of the sepsis pathology in this ER population, and was used to establish the cutoff level for plasma iNOS in this population of study participants. The physical status of each ER study participant was assessed based on retrospective medical record chart review. In the discovery cohort of ER study participants, 88 were admitted and 58 were not admitted, and of the 58 study participants not admitted, 22 (37.9%) were septic and 36 were not septic. The most disturbing aspect of these data involves the 22 individuals who were septic and went to the hospital seeking medical help but were sent home instead of being admitted (*54*). These sick individuals returned to the hospital within a couple of days very ill and unmistakably septic. Clearly, the healthcare system needs better IVD tests to help these very sick individuals. Because the background in the plasma iNOS assay was higher in plasma samples obtained from the ER patients compared with those obtained from ICU patients in previous studies, the cutoff value was increased from 1.5 ng/mL (5.0 fmol/mL) for ICU patients to 2.6 ng/mL (8.7 fmol/mL) for this patient population. Although the sensitivity and specificity for plasma iNOS as a biomarker for the onset of sepsis were lower in this patient population (Table 2) than previously found in ICU study participants, plasma iNOS was superior to PCT as a blood biomarker for the onset of sepsis (Table 2).

***ER clinical trial cohort #2.*** A validation clinical study on a second cohort of 154 ER patients was performed to further assess the ability of the plasma iNOS EIA test to detect extracellular MV-A iNOS in plasma as a biomarker for the onset of sepsis in ER patients. Again, the physical status of each ER study participant was assessed based on a retrospective medical record chart review. In this cohort of 154 ER patients, 118 were admitted and 36 were not admitted: Of the 36 study participants not admitted, 15 (41.7%) were septic. Again, the most worrisome aspect of these data involves the 15 individuals who were sent home instead of being admitted. When combined with the first ER cohort (Table 3), 37 of 300 (12.3%) septic study participants were not admitted to the hospital but were sent home. All 37 of these individuals returned to the hospital within a couple of days very sick and unmistakably septic. The plasma iNOS test detected 25 (67.6%) of these individuals as iNOS positive and at risk of becoming septic. As clearly shown in both cohorts of ER study participants, the healthcare system needs better diagnostic tools to help these very sick individuals. Other groups of investigators have also reported on the difficulty in assessing and differentiating the onset of sepsis in ER patients during triage because the symptoms

**Table 3. Summary triage results of ER study participants in combined cohorts 1 and 2.**

| Combined data for ER cohorts 1 and 2 n = 300 | | | |
|---|---|---|---|
| Admitted to hospital n = 206 | | Not admitted to hospital n = 94 | |
| Septic n = 139 | Not septic n = 67 | Septic[a] n = 37 | Not septic n = 57 |

[a] Each of these study participants returned to the hospital a few days after their initial visit and was admitted with a diagnosis of sepsis. When the plasma sample collected during their initial ER visit was assayed for plasma iNOS, 25 of these individuals (67.6%) yielded a positive iNOS test result.

for many inflammatory conditions are very similar (*55–57*).

## FUTURE POSSIBILITIES FOR A PANEL OF BLOOD BIOMARKERS FOR THE ONSET OF SEPSIS

The clinical data collected on both ICU and ER study participants strongly support the concept that plasma iNOS can serve as a standalone IVD test for the onset of sepsis. However, because many physicians prefer multiple pieces of information to help guide their clinical decisions, many research groups have explored numerous blood components in an attempt to identify a panel of biomarkers specific for sepsis (*58–61*). During the clinical studies on ICU patients discussed above, 3 additional components in blood from septic study participants were identified that (*a*) could provide additional important clinical information about an individual's sepsis status, (*b*) were not present in the circulation of nonseptic study participants or healthy individuals, and (*c*) appear as strong candidates for a multianalyte panel of biomarkers for the onset of sepsis—these are pro-interleukin-18 (pro-IL-18), pro-interleukin-33 (pro-IL-33), and regenerating protein 1-α (Reg1α) (*58*). The presymptomatic appearance of pro-IL-18 and pro-IL-33 appears to indicate an ongoing dysregulated immune response, and Reg1α emerged as an early presymptomatic biomarker for organ dysfunction. The pro-forms of the 2 cytokines are intracellular storage forms that are normally processed to mature cytokines before being released into the circulation, and as such the storage form is not normally present in extracellular compartments. Reg1α is normally involved in β-islet cell regeneration (*62*, *63*) and more recently has been proposed to serve as biomarker for bladder cancer (*64*). However, during the onset of sepsis, all 3 of these proteins have been found in plasma samples obtained solely from ICU study participants (*58*). Another proposed panel of biomarkers for sepsis includes C-reactive protein, PCT, neutrophils, macrophage migration inhibitory factor, soluble urokinase-type plasminogen activator receptor, and soluble triggering receptor expressed on myeloid cells-1 (*59*). A third proposed panel of biomarkers for sepsis includes PCT, interleukin-6, and soluble triggering receptor expressed on myeloid cells-1 (*60*), and a fourth proposed panel of biomarkers for sepsis includes 10 serum proteins of which 7 are proteins associated with inflammation (*61*). Depending on the complexity of the panel of biomarkers, the results of multianalyte tests are typically analyzed by an interpretive algorithm based on clinical trial data that provide a percentage likelihood of that patient being at an increased risk for sepsis. The results of the 4-biomarker panel that includes iNOS, pro-IL-18, pro-IL-33, and Reg1α for staging the onset of sepsis are interpreted using a straightforward and uncomplicated algorithm. Each of these panels should be tested individually and perhaps also in combination to identify the group of biomarkers that provides the best assay sensitivity and specificity for the onset of sepsis.

## LOOKING INTO THE FUTURE AND CONCLUDING REMARKS

At present, the standalone plasma iNOS test is transforming into a companion diagnostic test in front of a candidate mAb therapeutic to treat the onset of sepsis. Simultaneously, the plasma EIA test is being reformatted to a point-of-care test for use in resource-limited environments and field use. In the future, one or more multianalyte point-of-care test for sepsis will probably be developed for more prompt test results and increased accuracy and reliability.

Treatment paradigms such as early goal-directed therapy have not appreciably improved patient outcome for sepsis as demonstrated in 3 large-scale randomized clinical trials using several different primary and secondary end points (*65–67*). Meta-analyses of published clinical trial data have also been conducted that included data collected from large numbers of patients pooled into each meta-analysis (*68*, *69*). The results of these metaanalyses also demonstrated no statistically significant improvement in patient outcome with early goal-directed therapy as compared with normal standard-of-care procedures for sepsis (*68*, *69*). Thus, the clinical treatment of septic patients either by protocol or by community best practices yields similar clinical outcomes (*65–69*), and again highlights the need for better diagnostics and therapies for sepsis.

The only consistent factor in sepsis management that appears to have a positive impact on the clinical outcome is the early administration of antibiotics, which has been and remains the most important empiric therapeutic intervention for the management of sepsis, after resuscitation (*70*). However, the inappropriate use of antibiotics can lead to antibiotic resistance and overgrowth problems such as *Clostridium difficile* enterocolitis, a potentially fatal infection. Although PCT has been approved to guide antibiotic therapy (*71–73*), there is no accepted biomarker for initiating antibiotic treatment in patients with sepsis. A biomarker such as plasma iNOS that could both initiate and guide antibiotic administration for the sepsis pathology would be very impactful in clinical medicine. The clinical data gathered from ICU and ER patient populations strongly support the idea that an IVD for plasma iNOS can fulfill both of those roles because circulating MV-A iNOS appears to play a major role in the sepsis cascade. Plasma iNOS, therefore, should be more reliable than existing tests based on bystander molecules, assist in the stewardship of antibiotics by avoiding unnecessary antibiotic administration, and allow clinicians to monitor any necessary course of antibiotic treatment. Because a high percentage of sepsis cases are culture negative, it is important to have an accurate and reliable biomarker or panel of biomarkers for sepsis that will allow the prompt initiation of therapy and help guide the duration of the therapy (*3*, *74–76*).

Extracellular MV-A iNOS is a novel plasma biomarker for the onset of sepsis and appears to be intimately involved in the initiation of the sepsis cascade by producing toxic quantities of NO in susceptible receiver cells in the heart and vascular system and that comprise barrier structures in the body. The discovery of plasma iNOS has led to advances in the development of a targeted therapy by identifying those individuals who would benefit from an anti-MV-A iNOS mAb therapeutic. Numerous clinical trials on candidate sepsis therapeutics have failed primarily because of the absence of an appropriate diagnostic test in front of the candidate therapy (*77*, *78*). Several new potential blood biomarkers for sepsis have been recently described, and it is hoped that a well-defined biochemical definition of sepsis can be developed to accurately and reproducibly identify individuals who are becoming septic or who are already septic but presymptomatic for improved patient management and care. This should lead to a reduction in the economic and societal burden the sepsis pathology generates for healthcare systems worldwide.

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